Monocyte expression of tissue factor and adhesion molecules: the link with accelerated coronary artery disease in patients with chronic renal failure

N M Al-Saady, E W Leatham, S Gupta, J T C Kwan, J B Eastwood, C A Seymour

Abstract

***Objective*—To investigate the expression of monocyte tissue factor (MTF) and adhesion molecules in patients with chronic renal failure (CRF) and to look for any correlation with thrombin generation and Lp(a) lipoprotein.**

***Design*—A study of MTF expression and adhesion molecules, prothrombin fragments 1+2 (PTf1+2), an index of thrombin generation, and lipoproteins in patients with CRF and in normal control subjects.**

***Background*—Patients with end stage renal failure have an increased risk of coronary artery disease despite advances in therapy. Stimulated monocytes are potent activators of blood coagulation through the generation of MTF, which was recently implicated in the aetiology of acute coronary ischaemic syndromes.**

***Methods*—MTF expression and adhesion molecules were measured in whole blood using immunofluorescence of monocytes labelled with anti-tissue factor antibody and CD11b and c by flow cytometry. PTf1+2 and Lp(a) lipoprotein in plasma were measured by enzyme linked immunosorbent assay (ELISA).**

***Patients*—70 patients with CRF without documented coronary artery disease (30 patients with CRF undialysed, 20 patients undergoing chronic ambulatory peritoneal dialysis (CAPD), and 20 undergoing haemodialysis (HD)), together with 20 normal controls, were studied.**

***Results*—The (mean (SD)) increased MTF of CRF (48.0 (29) *v* 33.3 (7.2) mesf unit/100 monocytes in controls, p = 0.04) was more pronounced in patients undergoing dialysis (HD 73.1 (32.8) (p < 0.003) and CAPD 62.8 (28.9) mesf unit/100 monocytes, p < 0.04). MTF activity showed a positive correlation with both PTf1+2 and serum creatinine (p < 0.003) but not with Lp(a) lipoprotein. Lp(a) lipoprotein was significantly increased in both dialysis groups compared with controls (p < 0.005) and non-dialysis CRF groups (p < 0.02). Monocyte adhesion molecule (CD11b) was significantly higher in all three CRF groups than in the controls (p = 0.006).**

***Conclusion*—This study has demonstrated a hypercoagulable state in patients with CRF. This was especially pronounced in the dialysis patients. These findings provide a possible explanation for the increased cardiovascular and cerebrovascular morbidity and mortality in these patients.**

(*Heart* 1999;**81**:134–140)

Keywords: chronic renal failure; haemodialysis; continuous ambulatory dialysis; monocyte tissue factor; monocyte adhesion molecules; prothrombin; Lp(a) lipoprotein

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Accepted for publication
16 September 1998

Recent advances in therapy of patients with chronic renal failure (CRF) have been associated with improved survival and enhanced quality of life. Nevertheless, life expectancy is still considerably reduced. The dominant cause of this increased mortality is accelerated atherosclerosis[1] leading to cardiovascular death.[2] Many factors contribute to this premature atherosclerosis, and it is becoming apparent that lipoprotein abnormalities are closely associated with increased atherosclerosis risk and correlate well with cardiovascular mortality.[3]

Ritz *et al* noted the relative risk of fatal myocardial infarction to be 9–170-fold greater in dialysed uraemic patients than in the general population.[4] Samples from the renal and internal iliac arteries of uraemic individuals have shown unexpectedly advanced atherosclerosis and vascular aging compared with vessels from control populations.[5 6]

Acute myocardial infarction and cardiac death in the general population are associated with occlusive coronary thrombosis,[7 8] and it is probable that the enhanced thrombogenesis plays an important pathogenic role.[9] Indeed, several large epidemiological studies have shown increased concentrations of clotting factors VII, VIII, and fibrinogen to be independent risk factors for coronary artery disease.[9 10]

Monocytes (mononuclear white blood cells) are believed to play a key role in the development of the fatty streak, the earliest stage of atherosclerosis.[11–13] Leucocyte endothelial adhesion is important for experimentally induced atherosclerosis, and is abnormal in patients with hyperlipidaemia.[14] Monocyte adhesion molecule expression (mediated via integrin receptors) is increased in the coronary sinus blood of patients with unstable angina[15]; hence, the expression of monocyte adhesion molecules may be important in the pathogenesis of both acute and chronic phases of ischaemic heart disease.

Monocytes are known to exhibit pronounced procoagulant activity (PCA) in vitro when stimulated by endotoxin,[16] tumour necrosis factor,[17] and a variety of other immunological and inflammatory substances.[18–21] Similar PCA

has been described in monocytes isolated from animals injected with endotoxin[22] and in patients presenting with various medical disorders associated with hypercoagulability.[19 23–25] Recently monocytes from patients with unstable angina and myocardial infarction have been shown to have enhanced PCA and to correlate with markers of thrombin production.[26–28] Furthermore, macrophages removed from atherosclerotic plaques displayed similar properties in vitro.[29 30] It is therefore clear that monocyte PCA could be an important process in the aetiology of ischaemic heart disease. Evidence suggests that monocyte PCA is mediated by expression of a 47 kDa integral membrane glycoprotein (tissue factor (TF)) on the cell surface.[31 32] This glycoprotein binds to coagulation factor VIIa, initiating both intrinsic and extrinsic pathways of the coagulation cascade.[33 34]

Another risk factor for increased atherogenesis is Lp(a) lipoprotein, which also enhances PCA of peripheral blood mononuclear cells in vitro.[35] There is clinical evidence of an association between high Lp(a) lipoprotein concentrations and premature cardiovascular disease.[36] This association may also hold for patients with CRF, and we have shown in a pilot study that Lp(a) lipoprotein concentrations are significantly higher in chronic ambulatory peritoneal dialysis (CAPD) patients than in CRF patients.[37]

It is clear from the above that atherosclerosis development and enhanced thrombogenesis are important aetiological factors in the development of cardiovascular morbidity. As patients with CRF have such increased morbidity and mortality from cardiovascular and cerebrovascular events, it is clearly of interest to know if these factors operate in these patients. Our study provides evidence on the two pathogenic mechanisms, namely the development of atherosclerosis and the thrombogenic state.

Patients and methods

PATIENTS

Seventy patients with CRF—30 undialysed, 20 on CAPD, and 20 on haemodialysis (HD)—were studied. The CRF patients were recruited consecutively and had serum creatinine concentrations of 150–600 μmol/l. The CAPD and HD patients had been on dialysis for more than six months. They had not received heparin for at least 24 hours before blood sampling. Patients with malignancy, infection, existing known heart disease (angina or myocardial infarction), and diabetes mellitus were excluded from the study.

Twenty control subjects in good health, normolipidaemic, and receiving no drugs were selected from consecutive patients attending the outpatients' phlebotomy room for "screening" blood tests arranged by general practitioners.

SAMPLE COLLECTION

A venous sample was collected from each patient at between 08:00 and 10:00. The blood (2 ml) was drawn into a prechilled EDTA Vacutainer and kept on melting ice until analysed.

MEASUREMENT OF MONOCYTE TISSUE FACTOR EXPRESSION

A 100 μl volume of whole blood was incubated with 2 μg of rabbit antihuman TF and anti-CD11b and c antibodies (MoAb) for 30 minutes at 4°C. This rabbit antihuman antibody has been shown previously to have high specificity for human TF in western blot experiments, and blocks the functional activity of TF in procoagulant assays.

Red cells were lysed with Q prep solution (Coulter Corporation, Luton, UK), washed twice in chilled phosphate buffered saline, and then incubated with 1% paraformaldehyde before flow cytometric analysis.

Non-specific binding of antibodies was assessed for primary and secondary antibodies, using control antibodies (rabbit antichicken and swine antirabbit antibodies, respectively). These controls allowed determination of an appropriate threshold of surface fluorescence on unstimulated cells from normal subjects, above which only low levels (< 2.5%) of non-specific binding occurred.

FLOW CYTOMETRY

A Becton Dickinson flow cytometer (Facscan, Becton Dickinson, Mountain View, California, USA) was used to measure monocyte TF (MTF) expression. Leucocytes were gated on forward and side scatter. Pilot experiments showed the monocyte population to be CD14 (Coulter) positive (92 (5%)). Leucocyte surface fluorescence was measured using 10 000 cells for each sample.

After determination of TF expression in normal subjects, we set a threshold value of 2.5% TF positive cells, based on measurement of fluorescent activity without TF in standard samples. The flow cytometer was calibrated each day with calibration beads (CMPC, Hato Rey, Puerto Rico), which enabled the threshold for the detection of MTF to be standardised.

Lp(a) LIPOPROTEIN

Lp(a) lipoprotein concentration was measured using rate nephelometry in a Beckman Array 360CE. Rabbit antiserum (Dako Ltd, Cambridge, UK) was diluted 1 in 3 in buffer (Beckman buffer). Lp(a) lipoprotein calibrant (Immuno Ltd, Kent, UK) and samples were diluted 1 in 6 in apodiluent (Beckman apolipoprotein diluent) before analysis.

LIPID PROFILE

A standard lipid profile (total cholesterol, triglyceride, high density lipoprotein (HDL) cholesterol) was measured in the routine clinical biochemistry laboratory. Cholesterol and triglyceride were measured by enzymatic methods on the DAX 72 (cholesterol: method SM4 1139M90; and triglyceride: method SM4 1148M90). HDL cholesterol was assayed after dextran sulphate/magnesium chloride precipitation of low density lipoprotein (LDL) cholesterol followed by cholesterol analysis of the supernatant on a 12 Monarch centrifuge analyser. LDL cholesterol was determined by the Friedwald calculation.

Table 1 Mean (SD) lipid profiles in non-dialysed/dialysed patients with chronic renal failure and healthy controls

	Normal controls	*Chronic renal failure*	*CAPD*	*Haemodialysis*
Total cholesterol (mmol/l)	4.95 (0.9) (n = 20)	5.93* (1.5) (n = 25)	5.22 (1.5) (n = 19)	5.42 (0.9) (n = 15)
Triglyceride (mmol/l)	1.02 (0.4) (n = 20)	2.0* (1.0) (n = 25)	2.17 (1.6) (n = 19)	1.91 (1.0) (n = 15)
HDL cholesterol (mmol/l)	1.47 (0.4) (n = 20)	1.13* (0.05) (n = 23)	0.9* (0.3) (n = 12)	1.19* (0.4) (n = 11)
LDL cholesterol (mmol/l)	3.0 (0.8) (n = 20)	3.81* (1.0) (n = 23)	3.45* (1.2) (n = 12)	3.49* (0.7) (n = 11)

*p < 0.05.
CAPD, chronic ambulatory peritoneal dialysis; HDL, high density lipoprotein; LDL, low desnity lipoprotein.

STATISTICAL ANALYSIS

MTF comparisons between patients group and normal controls were made using the Kruskal-Wallis test for multiple group data and the *t* test. Except for the non-parametric analysis of variance test for a difference between groups (Kruskal-Wallis) which was one tailed, all tests were two tailed; $p < 0.05$ was considered significant. Multiple regression for MTF and prothrombin fragments 1+2 (PTf1+2) was used for the differences in age and sex between patients and normal controls. Because the distribution of Lp(a) lipoprotein data was highly skewed, results were expressed as median and range and non-parametric analysis using Mann-Whitney U test was carried out to test the significance between the groups. For the lipid profile, a mean difference was tested using the unpaired *t* test. Pearson correlation coefficients for Lp(a) lipoprotein, C reactive protein, and serum creatinine were also tested.

Results

The results of the lipid profiles are presented in table 1. Patients with CRF (undialysed) showed significantly higher concentrations of total cholesterol, triglycerides, and LDL cholesterol, and lower concentrations of HDL cholesterol, compared with the controls. CAPD and HD patients showed a similar trend but concentrations were only significantly different from controls for triglycerides and HDL cholesterol.

ACUTE PHASE PROTEIN

There were no significant differences in C reactive protein, α_1 antitrypsin, and haptoglobin between the groups.

MONOCYTE TISSUE FACTOR EXPRESSION

Figure 1 shows representative histograms of monocyte surface MTF expression in one patient and one normal control. Increased MTF expression was observed in the dialysis and non-dialysis groups compared with normal controls (fig 2). Monocyte MTF expression (mesf unit/100 monocytes) was significantly raised in patients with CRF compared with controls (mean (SD)) (48 (29) *v* 33 (7.2); p = 0.04). Furthermore, MTF expression was particularly high in those undergoing HD and CAPD (73.1 (32.8), p = 0.003, and 62.8 (28.9), p = 0.04, respectively), with no significant difference between the two dialysis groups (fig 2). The percentage of monocytes which showed expression of MTF was significantly higher in the dialysis and non-dialysis groups (51% and 43%, respectively) compared with the controls (18%, p = 0.013).

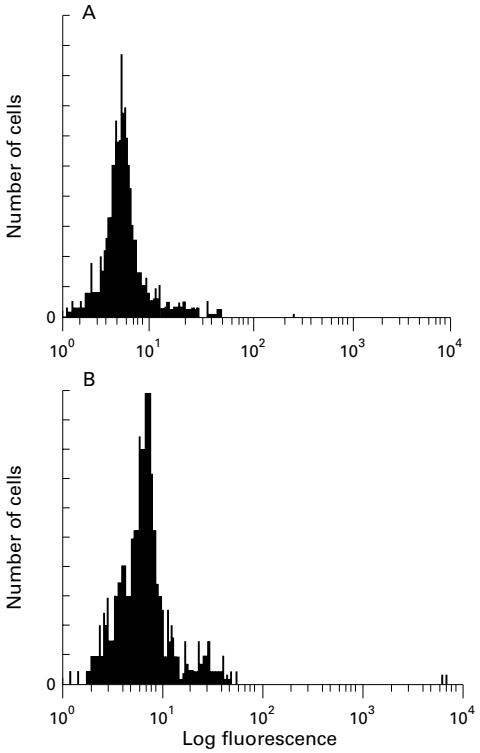

Figure 1 Histograms of fluorescence intensity (log scale). (A) Normal control. (B) Chronic renal failure patient.

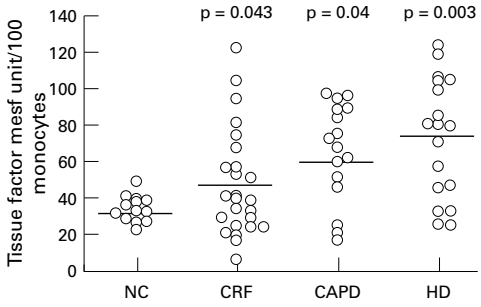

Figure 2 Monocyte tissue factor (MTF) expression in normal controls (NC) and chronic renal failure (CRF) patients. Data show individual's mean. p values represent comparison with NC. CAPD, chronic ambulatory peritoneal dialysis; HD, haemodialysis.

Multiple regression model for MTF showed that the difference between patients and normal controls was 20.5 mesf unit/100 monocytes (95% confidence interval (CI) 2.2 to 35.7, p < 0.03) after adjustment for age.

PROTHROMBIN FRAGMENTS 1+2

PTf1+2 were significantly higher in the non-dialysis CRF (1.7 (0.6) nmol/l) and dialysis groups (HD 3.2 (1.5) nmol/l, CAPD 2.3 (0.8) nmol/l) than in controls (0.86 (0.3) nmol/l, p < 0.001), with no significant difference between the two dialysis groups (fig

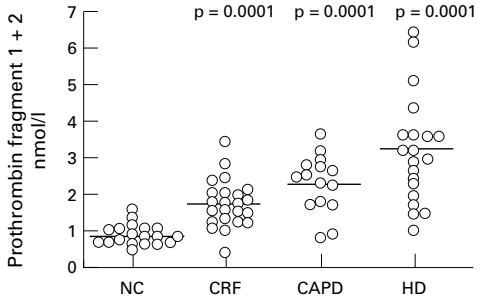

Figure 3 Prothrombin fragments 1+2 (PTf1+2) in normal controls (NC) and chronic renal failure (CRF) patients. Data show individual's mean. p values represent comparison with NC.

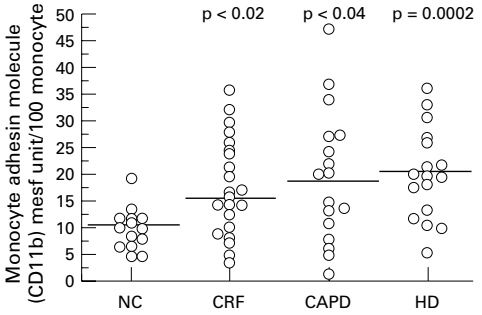

Figure 4 Monocyte adhesion molecules in normal controls (NC) and chronic renal failure (CRF) patients. Data show individual's mean. p values represent comparison with NC.

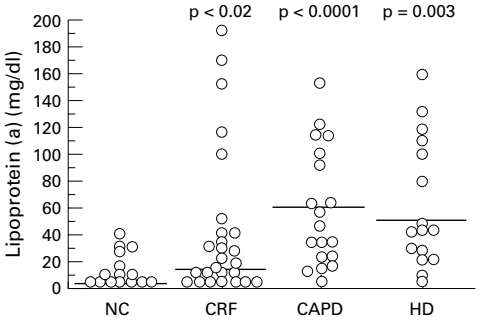

Figure 5 Lp(a) lipoprotein in normal controls (NC) and chronic renal failure (CRF) patients. Data shows individual's median. p values represent comparison with NC.

3). There was a positive correlation between MTF expression and PTf1+2 ($p < 0.004$).

The multiple regression model for PTf1+2 showed that the difference between patients and normal controls after adjustment for age was 1.39 nmol/l (95% CI 0.8 to 1.88, $p = 0.0001$).

It was not possible to differentiate the effects of underlying renal disease on MTF or PTf1+2 because of the wide variation in the causes of renal failure. However, both MTF and PTf1+2 showed significant correlations with serum creatinine in the undialysed CRF patients ($p = 0.0001$ and 0.0001, respectively). This suggests that the severity of the renal failure is positively associated with influence on MTF and PTf1+2.

ADHESION MOLECULES

Adhesion molecule CD11b (mesf unit/100 monocytes) was significantly higher ($p = 0.006$) in dialysis and non-dialysis groups compared with normal controls (mean (SD): for normal controls 10.2 (3.6), for undialysed CRF patients 16.5 (9.2), for CAPD patients 18.4 (12), and for HD patients 21.1 (8.5)) (fig 4). CD11c, although showing a similar trend to CD11b, did not reach statistical significance. CD11c (mesf unit/100 monocytes) in normal controls was 8.6 (3.1), while in undialysed CRF patients it was 11.8 (8), in CAPD patients it was 13.4 (10.8), and in HD patients it was 12.0 (4).

CD11b showed a positive correlation with CD11c ($p = 0.0001$) and serum creatinine ($p = 0.0001$). Both MTF and PTf1+2 showed a positive correlation with CD11b ($p = 0.003$, $p = 0.0001$, respectively).

Lp(a) LIPOPROTEIN

Lp(a) lipoprotein serum concentrations were significantly higher in dialysis and non-dialysis patients than in controls ($p < 0.003$): median (range) for normal controls 6.2 (5.0–41.5) mg/dl, for undialysed CRF patients 16.2 (5.0–192.5) mg/dl, for CAPD patients 47.5 (5.0–153.2) mg/dl, and for HD patients 42.6 (5.0–160.0) mg/dl (fig 5). Lp(a) lipoprotein showed a positive correlation only with serum creatinine ($p = 0.01$); no significant correlation was found between Lp(a) lipoprotein and MTF or PTf1+2.

Discussion

In this study we show for the first time that MTF expression is increased in dialysis (CAPD and HD) and non-dialysis CRF patients compared with healthy control subjects, with the highest values being in the dialysis groups. Another new finding is that increased monocyte MTF expression is associated with a significantly raised serum concentration of PTf1+2 (an index of thrombin generation). There was a fourfold increase in PTf1+2 in the HD group, a threefold increase in the CAPD group, and a twofold increase in the non-dialysis group compared with the control group. Furthermore, monocyte integrin (adhesion molecule CD11b) expression was significantly increased in the non-dialysis and dialysis groups compared with the control group.

Renal abnormalities may cause both a bleeding diathesis and a hypercoagulable state.[38 39] In patients on regular HD, haemostatic abnormalities include an increase of coagulation factors (fibrinogen and FVIIc),[40 41] low concentrations of coagulation inhibitors (protein C, antithrombin III, and heparin cofactor II),[38 41–44] and an increase of molecular markers of coagulation activation (thrombin-antithrombin II, PTf1+2, plasmin α_2-plasmin inhibitor complex, and D-dimer).[40 41] Raised plasma concentrations of thrombomodulin and von Willebrand factor have also been reported,[41 45–47] perhaps indicating endothelial cell injury in dialysis patients. These abnormalities, in addition to lipid abnormalities,[48] may explain in part the high incidence of cardiovascular death in chronic HD patients. In the present study, we found that MTF

expression by monocytes correlated positively with thrombin generation (indicated by measured PTf1+2), the combination of which would be a potential risk for cardiovascular disease. This new evidence indicates that patients on regular dialysis have a hypercoagulable state.

MTF expression was measured using flow cytometry. This technique is used to measure immunofluorescence of cells labelled with appropriate antibodies and is able to detect specific cell surface receptors as markers. The technique has been accurately used for the assessment of leucocyte intern (adhesion molecules) and to study monocyte MTF expression.[28 49] Luther[49] showed that MTF expression, determined cytometrically, had significant correlation with in vitro PCA. Furthermore, direct measurement of monocyte MTF with this method is more sensitive than PCA and is less likely to be influenced by PCA inhibitors. Flow cytometric measurement of monocyte MTF expression is usually carried out using Ficoll separated monocytes.[49] Although a whole blood technique[50] is now common practice for labelling blood cells with other antibodies, we were the first to describe a whole blood method for assessing monocyte MTF expression in coronary artery disease patients in a previous study,[28] and in patients with CRF in the present study (fig 1). This method has the advantage of reducing the time taken to label monocytes. Furthermore the rapid incubation at 4°C reduces the chance of sample contamination with endotoxin, which might cause an artefactual increase in MTF expression.

Neri Serneri and colleagues[26] have shown that separated mononuclear cells show increased procoagulant or "TF-like" activity in cultured monocytes from patients with unstable angina compared with convalescent and normal control samples. One other report of increased monocyte related PCA in unstable angina has been published in abstract form.[27] Our previous[28] and present studies indicate that such mononuclear cell procoagulant effects are mediated, in part, by TF expressed on monocytes.

TF, an integral membrane protein located on the surfaces of certain cell types, is generally held to be the physiological trigger of coagulation in normal haemostasis. It activates both intrinsic and extrinsic coagulation pathways. Thus expression of MTF on circulating monocytes is likely to result in an enhanced systemic coagulant state, which may have a role in atherothrombosis. Monocytes are the only circulating blood cells capable of expressing MTF. Furthermore, as monocyte adhesion, migration, and transformation into tissue macrophages is known to occur at the site of atherosclerotic plaque, focal production of TF may explain the propensity for thrombosis at the site of an atheromatous plaque. CRF monocytes may therefore provide a useful model for further investigation of the increased atherogenesis and thrombosis which occurs in CRF despite reduction in urea and other "uraemic" toxins by treatment.

There are only two published studies of MTF in CRF.[52 53] Unlike our study, both groups measured plasma TF rather than monocyte MTF expression. Like us, however, they found increased plasma TF in the patients with CRF and even higher concentrations in their HD patients. Our study is likely to be more clinically relevant, in that we have measured monocyte MTF rather than plasma TF expression in the cell intimately involved in atherogenesis. Furthermore, unlike these two studies,[52 53] we have measured the impact of the increased MTF activity on thrombogenicity (PTf1+2) and found that it correlates well ($p < 0.003$).

Conversely Koyama *et al* reported that plasma TF concentrations were increased in uraemic patients on chronic dialysis, while remaining within the normal range in non-dialysis uraemic patients.[52] Differences between these two studies may be explained in part by the different antibodies used in the enzyme linked immunosorbent assay (ELISA) systems.

Kario *et al* reported in a separate study that plasma TF pathway inhibitor (TFPI) activity was also considerably increased in uraemic patients.[54] TFPI is a powerful inhibitor of the factor VIIa/TF complex in the presence of factor Xa, as well as being a direct inhibitor of FXa.[55 56] The high concentration of TFPI in their study might thus act as a balance to the increase of FVIIa in uraemia. However, in our study PTf1+2 was used as an index of thrombin generation rather than FVIIa or TFPI. PTf1+2 plasma concentrations were considerably increased in dialysis and non-dialysis groups, indicating that monocyte MTF expression was not balanced by the generation of TFPI.

Monocyte MTF expression can be induced in vitro by a variety of stimuli including soluble immune complexes,[57] lymphokines,[58] lectins,[59] endotoxins,[49] viruses,[60] and chemically modified LDL.[61] The cause of increased monocyte surface MTF and integrin expression in patients with CRF is currently uncertain. One possible explanation is that they are an acute phase response to tissue damage. We think this is unlikely, as other components of this response (C reactive protein, α_1 antitrypsin, and haptoglobin) were normal in our CRF patients. In our study monocyte MTF and integrin expression as well as PTf1+2 and Lp(a) lipoprotein correlated positively with serum creatinine, which indicates that renal impairment itself may contribute to these raised concentrations.

Our findings similarly suggest that monocyte MTF expression may be less transient than is generally accepted. The increased expression in these patients together with thrombin generation indicates a hypercoagulable state in which monocytes may play a major role. Furthermore the increased expression of monocyte integrin might indicate a continuous or intermittent stimulation or trigger leading to their activation. Tissue damage and necrosis in the region of an atheromatous plaque may stimulate circulating monocytes and account

for the increased expression of both adhesion molecules and MTF found in our study. Alternatively, or in addition, an increased monocyte MTF expression may precede acute coronary syndromes and may predispose to their development.

The patients in our study had no manifestations of ischaemic heart disease. Furthermore not all patients showed an increased percentage of MTF positive monocytes. Almost half the monocytes showed an increased expression of MTF compared with only 18% in the healthy control subjects. This finding does not invalidate our hypothesis that monocytes initiate thrombosis, as MTF expression is only one of several mechanisms by which these cells may induce thrombosis. Monocytes have been shown to produce several constituents of the clotting cascade, including factors V, VII, VIII, Mac-1 dependent factor X, and prothrombinase.[62 63]

The changes in Lp(a) lipoprotein, which we have noted in this study and previously,[37] although not showing any correlation with monocyte expression of MTF or integrin, would also suggest an independent thrombogenic role. This, together with the finding of dyslipidaemia (an increase in the atherogenic lipoproteins, LDL cholesterol and triglyceride, and a decrease in the protective lipoprotein, HDL cholesterol) in these patients are further evidence of an increased risk of atherosclerosis and its acceleration.

Our study did not look at markers of endothelial dysfunction/injury, such as thrombomodulin and von Willebrand factor, to see if they had any relation to our findings. Investigation of monocyte MTF, integrin, and PTf1+2 in CRF patients with ischaemic heart disease will be needed in further studies. However, this study demonstrates that CRF monocytes, because of the increased expression of adhesion molecules, are a good model for studying the activity and role of monocyte MTF expression as a coronary artery disease risk factor.

In summary, this study shows that circulating monocytes from CRF patients exhibit increased expression of MTF and integrin. The positive correlation with thrombin generation suggests that their blood is hypercoagulable. Furthermore, these patients have increased serum concentrations of atherogenic lipoproteins, especially Lp(a) lipoprotein, which puts them at an increased risk of accelerated atherosclerosis/increased thrombogenicity. These abnormalities are of particular interest because they persist despite treatment, and are even more pronounced in the dialysed patients. Clearly any specific therapy for preventing atherosclerosis in these patients needs to be directed not only at the hypercoagulable state but also at monocyte adhesion.

We would like to thank Dr Jan Poloniecki for his invaluable expert advice in planning the study, statistical analysing and reading the manuscript. This work is fully supported by a British Heart Foundation project grant (BHF-PG94153).

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